Review—The Use of Immunosuppressive Agents to Prevent Neutralizing Antibodies Against a Transgene Product

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ABSTRACT: A potential obstacle to successful gene therapy for some patients is the *in vivo* production of neutralizing antibodies against the recombinant therapeutic product delivered. This is a problem inherent to all gene therapy methods, regardless of the vector used to deliver the protein. This clinical situation can be mimicked in animal models by delivering a foreign protein (i.e., a human protein) to the animal to provoke anti-human protein antibody production. The efficacy of different immunosuppressive treatments to inhibit the development of neutralizing antibodies can then be investigated. The immunosuppressive agents examined here include drugs (e.g., cyclophosphamide, FK506), cytokines (e.g., interferon-γ, interleukin-12), and monoclonal antibodies (e.g., anti-CD4, anti-gp39, CTLA4-Ig). It has been found that a high level of antibody suppression is necessary to allow prolonged delivery of a foreign protein. Immunosuppressive agents capable of this high level of suppression will be important adjuncts to prevent treatment failures in situations where patients are at risk of developing neutralizing antibodies.

INTRODUCTION

Many genetic diseases, which are caused by the absence of a functional protein, are potentially amenable to treatment by gene therapy protocols. However, protein replacement therapy for some of these disorders has revealed that neutralizing antibodies can form against the therapeutic product. Approximately 10–15% of hemophilia A[1] and 2% of hemophilia B[2] chronic transfusion patients develop antibodies to factor VIII or IX, respectively, which inhibit clotting functions and decrease the efficacy of the replacement therapy. Similarly 13% of patients[3,4] with Gaucher disease, one of the more than 30 lysosomal storage disorders, developed antibodies against the enzyme glucocerebrosidase used for replacement therapy. Even in animal models of many lysosomal storage disorders, development of antibodies against the therapeutic product is common. For example, in the mucopolysaccharidosis (MPS) type I dog deficient in α-iduronidase[5] and the MPSVII mouse deficient in β-glucuronidase,[6] the development of antibodies to the replacement protein has been well documented. In addition to loss of efficacy, anaphylaxis during subsequent treatments is a potential clinical complication. Unfortunately, it appears that the more se-

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verely affected the patients, the greater tendency for them to become sensitized against the replacement protein as a result of their cross-reacting-material-negative phenotype. Thus, for patients who need the therapy most may have a higher risk of becoming resistant to the treatment.

Other sources causing immunological response from the host include the viral vectors used in gene therapy. The vector, usually a recombinant virus or plasmid, is genetically engineered to contain the gene of interest, along with various regulatory sequences, such as promoters, enhancers, and genes used to select for transfected cells (e.g., antibiotic-resistance factors or cell surface markers). The most common vectors used are retroviruses, followed by plasmids, adenoviruses, pox-viruses, and adeno-associated viruses. Vectors which elicit a host inflammatory or immune response are less likely to be able to sustain delivery of the trans-gene product. Viruses have numerous proteins which can stimulate the immune system. Adenoviruses are strong activators of host inflammatory and immune responses (cellular and humoral) leading to loss of transgene delivery.[7] Newer adenoviral constructs with fewer viral proteins have been shown to decrease T-cell response. The most recent development is adenoviral vectors with no viral proteins at all,[8] or with co-expression of anti-inflammatory cytokine to avoid inflammatory and immune responses.[9] A commonly used retrovirus is the Moloney murine leukemia virus (MLV), which has the advantage that it does not elicit a host inflammatory/immune response.

Non-viral methods usually use recombinant plasmid DNA to deliver the target gene to the host cell. Compared to viral transduction, these methods generally have poor efficiency. However, depending on the delivery method, the host inflammatory response can be avoided. In our approach, a nonautologous universal cell line (mouse C2C12 myoblasts) containing a plasmid engineered to produce human growth hormone (hGH) was placed in immuno-protective alginate-poly-L-lysine-alginate microcapsules. The microcapsules were protected from immune rejection after intraperitoneal implantation into the allogeneic hosts, C57Bl/6 mice, thus allowing a continuous delivery of hGH *in vivo* by the encapsulated cells.[10] Owing to the size exclusion property of the microcapsule membrane, the larger immune mediators, likely including complement and lymphocytes, are excluded from entry, while passage remains unimpeded for the smaller molecules such as nutrients, metabolic waste products and the transgene product, with molecular weights below the cut-off threshold.[11] While immunoisolation protected the encapsulated cells from rejection, the immune system was fully capable of reacting to the recombinant protein secreted from the encapsulated cells.[12] This method of gene product delivery provides a useful model to mimic the clinical complication of neutralizing antibodies in gene therapy and to develop strategies to suppress such immune response.

PRINCIPLE OF IMMUNOSUPPRESSION

Stimulation of T-cell dependent antibody production is a complex process (FIG. 1). Antigen-presenting cells (APC) such as macrophages, dendritic cells, and B-cells internalize foreign antigens and then present them on their cell surface in association with the major histocompatibility complex (MHC) class II. This complex is recognized by the CD4–T-cell receptor (TCR) on T-helper cells, but a second signal is re-

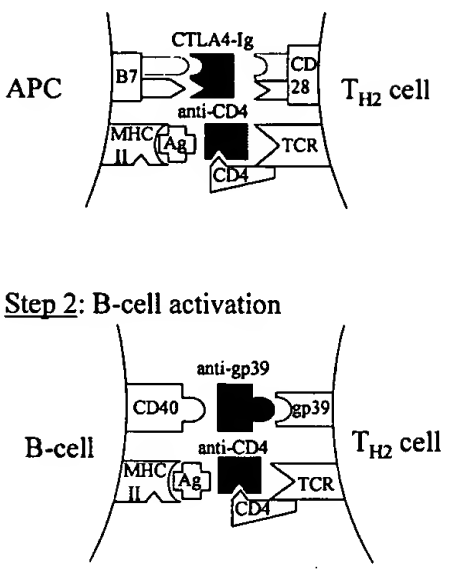

FIGURE 1. Immune signaling for antibody production and then immune suppressive agents. ***Step 1***: Antigen presenting cells (APC) present foreign antigens (Ag) along with the major histocompatibility complex type II (MHC-II) to T-helper cells. This signal is recognized by the T-cell receptor (TCR) and CD4 molecule on the T-helper cell. Activation of the T-helper cell also requires a second signal, the B7–CD28 interaction. If the APC secretes IL-12 (e.g., if it is a macrophage), the activated T-helper cells preferentially differentiate into IL-2, IFN-γ, and tumor necrosis factor (TNF) β–secreting T_{H1}, cells instead of the T_{H2} cells which are more important in antibody production. The immunosuppressive agents which act during this step are FK506, CTLA4-Ig, anti-CD4 antibodies, IL-12 and IFN-γ (see TABLE 1 for details). ***Step 2***: The activated T_{H2} cell then activates B-cells via the MHC-II restricted recognition of the antigen, also requiring a second signal, the gp39–CD40 interaction. The T_{H2} cells secrete IL-4, -5, -6, -10, and -13 to stimulate B-cell proliferation and differentiation into antibody-producing plasma cells. The immunosuppressive agents which act during this step are cyclophosphamide and anti-gp39 (see TABLE 1 for details).

quired for activation of the T-cell. A potent costimulator is the B7 molecule on the APC, which binds to the CD28 molecule on the T cell, stimulating T-cell proliferation. These activated T-helper cells can then stimulated resting B-cells by MHC-II restricted antigen interaction with the CD4-TCR on the T-helper cell. A costimulatory interaction between CD40 on the B-cell and gp39 (CD40 ligand) on the T-cell ensures B-cell activation. Activated B-cells are then stimulated into clonal proliferation and to differentiate into immunoglobulin producing plasma cells.[13]

Several methods of immune suppression may be potentially useful in attenuating the formation of neutralizing antibodies. They include the use of general immunosuppressive drugs or specific immune modulators that block the various pathways leading from antigen presentation to T- and B-cell activation (TABLE 1). The immunosuppressive agents examined here include the immunosuppressive or cytotoxic drugs cyclophosphamide and FK506 (group I). As well, immune signal blocking

TABLE 1. Immunosuppressive agents and their possible mechanisms of action

Agent	Mechanism	Reference
Group I: Immunosyppressive drugs		
cyclophosphamide	prevents clonal expansion of B cells and antibody expression	Dai *et al.*, 1995[14]
FK506	interferes with T-cell differentiation and proliferation	Lochmuller *et al.*, 1996[15]
Group II: Antibodies		
CTLA4-Ig	competitive inhibitor of CD28	Abbas *et al.*, 1994[17]
anti-gp39	blocks the interaction between the gp39 protein and the CD40 B-cell receptor	Foy *et al.*, 1993[20]
anti-CD4	transiently depletes CD4 cells	Yang *et al.*, 1996[21]
Group III: Cytokines		
interleukin-12	induction of T_{H1}clones	Paul and Seder, 1994[24]
interferon-γ	inhibits proliferation of T_{H2} clones	Paul and Seder, 1994[24]

monoclonal antibodies such as anti-CD4, anti-gp39, CTLA4-Ig (group II) and immune mediators such as the cytokines interferon-γ and interleukin-12 (group III) are also examined.

EXPERIMENTAL METHODS

Recombinant Cells for Encapsulation

A mouse C2C12 myoblast cell line (C41) was transfected with the plasmid pNMG3. The pNMG3 plasmid coded for the *hGH* gene driven by the mouse metallothionein-1 promoter and the *NeoR* gene conferring resistance to G418. Prior to encapsulation, cells were maintained in Dulbecco's medium supplemented with 10% fetal bovine serum (FBS) and Penicillin (100 units/mL), streptomycin (100 μg/mL) and G418 (400 μg/mL). Immediately following encapsulation and washing, the cells were maintained in SkBM low-serum medium (Clonetics, San Diego, CA) until implantation.

Encapsulation

The cells were encapsulated in alginate-poly-L-lysine-alginate microcapsules. Briefly, cells harvested with trypsin were resuspended in 1.5% potassium alginate (Improved Kelmar, Lot 17703A, Kelco, San Diego, CA) at 2×10^6 cells/mL, extruded with a 27G blunt end needle as droplets into 1.1% $CaCl_2$ to form gelled beads, coated with poly-L-lysine and alginate to form the outer membrane, and the core solubilized with sodium citrate to form microcapsules enclosing the recombinant cells. The microcapsules were kept in SkBM low serum medium until implantation.

Animals

Male C57Bl/6 mice (3–4 week old, ~15 g body weight) were housed in pathogen-free housing with micro-isolator hooded cages. All procedures were performed under a laminar flow hood with sterile techniques and in accordance with Canadian Institutional Animal Care Guidelines.

Capsule Implantation

The animals were briefly anesthetized with a small-animal anesthetic machine providing a controlled amount of isofluorane and oxygen. Approximately 3 mL of washed microcapsules were implanted into the peritoneal cavity of each mouse with a 16G catheter. Baseline blood sampling and body weight measurement were performed immediately before the implantation. The procedure took 10–15 min and the animals were soon freely mobile in their cages.

Blood Collection

Retro-orbital bleeds were conducted on anesthetized mice on days 0, 2, 7, 14, 21, and 28 post-implantation. Approximate 100 μL of whole blood was collected with a heparinized capillary tube and plasma stored at –20°C until assayed. On day 28, one mouse from each group was sacrificed via terminal cardiac bleed for retrieval of microcapsules.

Capsule Retrieval

A full midline incision of the abdominal skin and a 0.75 cm incision through the abdominal wall were made to retrieve the capsules from the peritoneal cavity. They were washed three times with ice-cold PBS and then with supplemented Dulbecco's medium. The capsules were maintained in this medium under the usual culture conditions

Clearance Study

Three animals from a treatment group (CTLA4-Ig) sensitized to hGH, owing to microcapsules implanted 22 weeks previously, and four naïve mice of similar weight and sex were each injected into the tail vein with 6 μg of purified hGH (gift from Eli Lilly Inc., Indianapolis, IN). Blood was sampled by retro-orbital bleeds at 1, 3, 5, 10, 15 and 30 min after the injection. The mice were sacrificed after the final blood sample.

Reagents and Treatment Protocols

Four mice were used for each treatment. Two mice died on day 1 following implantation (one each from the FK506 and IFN-γ treatments).

Controls[e]

- Negative control: received only microcapsules but no immune suppressive treatments.
- L6 (a miscellaneous fusion protein as a matched negative control for CTLA4-Ig, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA): 200 μg IP (1 mg/mL saline) q2d × 5 doses starting on day 0 of implantation (total dose 1000 μg/mouse).

Group I: Immunosuppressive Drugs

- Cyclophosphamide (Carter-Horner, Mississauga, Ont., Canada): 250 mg/kg injected IP (20 mg/mL), one dose on day −1 prior to implantation.
- FK506 (Fujisawa USA, Inc., Deerfield, IL, USA): 5 mg/kg SC (0.5 mg/mL saline) daily starting at day −2 prior to implantation.

Group II: Monoclonal Antibodies

- CTLA4-Ig (Bristol-Myers Squibb, Pharmaceutical Research Institute, Seattle, WA): 200 μg IP (1 mg/mL saline) q2d × 5 doses starting on day 0 of implantation (total dose 1000 μg/mouse).
- Anti-gp39 (Pharmingen Canada, Ontario, Canada): 250 μg IP q2d × 3 doses starting on day 0 of implantation (total dose 750 mg/mouse).
- Anti-CD4 (Pharmingen Canada, Ontario, Canada): 250 mg IP q3d × 3 doses starting on day −3 prior to implantation (total dose 750 μg/mouse).

Group III: Cytokines

- Interferon-γ (Pharmingen Canada, Ontario, Canada): 2 μg IP (10 μg/mL PBS) on day 0 and day 1 post-implantation (total dose 4 μg/mouse).
- Interleukin-12 (Hoffmann-LaRoche, Nutley, NJ): 2 μg IP (1 mg/mL PBS) on day 0 and 1 post-implantation (total dose 4 μg/mouse).

Human Growth Hormone Assay

An ELISA kit (UBI-Magiwel hGH kit, United Biotech, CA) was used according to the supplier's instructions. The rate of hGH secretion from capsules was determined by sampling aliquots of the media at 0, 1, 2, and 4 hours. Mouse plasma levels were determined at 1:3 dilutions. The limit of detection for the assay was 0.2 ng/mL, which gave a limit of detection of 4 ng/mL for the rate of secretion assay and 0.6 ng/mL for the plasma assay.

Human Growth Hormone Antibody Assay

Antibodies to hGH were assayed with an ELISA protocol as follows, washing 3 times with PBS/Tween (10 mM Na_2HPO_4, 150 mM NaCl, 0.05% Tween 20, pH 7.4)

[e]Abbreviations: q2d–every two days; q3d–every three days; OD–once daily; IP–intraperitoneal; SC–subcutaneous.

between steps. Each well was coated with 200 ng pure hGH (Humatrope, supplied by Eli Lilly Inc. Indianapolis, IN) for 2 h at 37°C, blocked with 5% skim milk powder in PBS/Tween for 2 h at 37°C, treated with 100 μL plasma sample diluted 1:300 in 5% skim milk powder in PBS/Tween at 37°C for 1 h, incubated with goat anti-mouse antibody conjugated to alkaline phosphatase (Promega, Madison, WI) at 37°C for 1 hr, and then detected with Sigma 104 Phosphatase substrate. A standard curve was constructed by making serial dilutions serum from a positive control mouse that was immunized with pure hGH. All sample antibody titers were expressed as the equivalent dilution of the positive control serum to contain the same amount of anti-hGH antibodies.

Statistical Analysis

ANOVA was carried out to detect significant differences between the groups at each time point. When a significant difference was detected ($p < 0.05$), a Scheffe test was performed to identify the significant groups.

SUPPRESSION OF IMMUNE RESPONSE IN A MODEL OF GENE THERAPY

Using the murine model for nonautologous gene therapy, we delivered hGH to mice by intraperitoneal implantation of microencapsulated recombinant cells. Plasma hGH levels and the antibody response to hGH in control mice were then compared to those in mice treated with one of the immunosuppressive agents. In the control mice, the circulating hGH was clearly detectable by day 2 post-implantation, reaching a peak level of ~2 ng of hGH/mL plasma on day 7, but declining to background level by day 14 (FIG. 2A, left panel). As expected, the xenogeneic hGH, being a foreign antigen, provoked an immunological response, leading to a corresponding rise in anti-hGH antibodies, which were first detectable by day 14 and continued to increase until day 28 (FIG. 2A, right panel). When the implanted mice were treated with the three groups of immunosuppressive agents, their responses were highly variable, depending on the protocol used (TABLE 2).

Group I

These mice were treated with one of the two immunosuppressive drugs, cyclophosphamide or FK506. The delivery pattern was similar to that of the controls, peaking on day 7 at 2.0 ng/mL and 0.9 ng/mL plasma, respectively, before subsiding to background level by day 14 (FIG. 2B, left panel). However, both drugs in this group were able to down regulate the antibody response to hGH when compared to the untreated controls. The cyclophosphamide-treated mice showed only a low titer response beginning on day 14, while FK506 did not show any antibody response until day 21 (FIG. 2B, right panel). Cyclophosphamide is used primarily as an anti-cancer drug while FK506 (Tacrolimus, Prograf™) is a newer drug that has been used as an anti-rejection agent in organ transplants as well as in somatic gene therapy models to suppress host antibody response. Treatment with cyclophosphamide prevents clonal expansion of B cells and antibody expression[14] whereas FK506 is an antibi-

TABLE 2. Treatment groups and growth of encapsulated cells retrieved on day 28 post-implantation[a]

Treatment	Protocol	% Cell Viability	No. Viable Cells/Capsule
In Vitro			
Day 0	N/A	94 ± 5	231 ± 64
Day 7	N/A	93 ± 1	185 ± 1
Day 14	N/A	83 ± 2	425 ± 158
Day 28	N/A	80 ± 7	302 ± 91
In Vivo			
Controls:			
Negative control (N = 4)	no treatment	87 ± 1	999 ± 370
L6 (N = 4)	200 µg IP q2d on days 0 to 8	69 ± 11	1230 ± 617
Group I: Immunosuppressive drugs			
Cyclophosphamide (N=4)	250 mg/kg IP on day −1	89 ± 5	1430 ± 551
FK506 (N = 3)	5 mg/kg SC daily	94 ± 2	2360 ± 465
Group II: Antibodies			
Anti-gp39 (N = 4)	250 µg IP q2d on days 0 to 4	84 ± 3	1390 ± 383
Anti-CD4 (N = 4)	250 µg IP q3d on days −3 to 3	83 ± 5	1150 ± 141
CTLA4-Ig (N = 4)	200 µg IP q2d on days 0 to 8	84 ± 4	1870 ± 845
Group III: Cytokines			
IFN-γ (N = 3)	2 µg IP on days 0 and 1	88 ± 4	1420 ± 171
IL-12 (N = 4)	2 µg IP on days 0 and 1	83 ± 12	2500 ± 351

[a]Encapsulated hGH-secreting cells were either kept *in vitro* for 28 days or implanted intraperitoneally into mice treated with various immunosuppressive reagents according to the different protocols. On day 28 post-implantation, the microcapsules were retrieved with intraperitoneal lavage to determine the viability and number of encapsulated cells. The data are means ± standard error. Abbreviations: IP–intraperitoneal; SC–subcutaneous; q2d–every second day, q3d–every third day. (From Potter *et al.* 1998,[26] reprinted with permission.)

otic produced by *Streptomyces tsukubaenis* that interferes with T-cell differentiation and proliferation.[15] They both showed good suppression of antibody response (11 ± 3% and 6 ± 5% of the control, FIG. 2). In other experiments, cyclophosphamide had been used successfully at a dose of 300 mg/kg to prevent the formation of anti-adenovirus–neutralizing antibody in C57Bl/6 mice,[16] thus allowing a repeat administration of human factor IX via an adeno-viral vector. We were not able to achieve total antibody suppression at a dose of 250 mg/kg cyclophosphamide, and a dose of 300 mg/kg cyclophosphamide was poorly tolerated in a pilot study (unpublished data). Similarly, FK506 had been used to suppress antibody response to dystrophin and an adenovirus vector, allowing dystrophin transgene expression for more than 2 months.[15] In our study, FK506 was an effective agent in suppressing the anti-hGH antibody response (FIG. 3), thus verifying the effectiveness of this drug treatment.

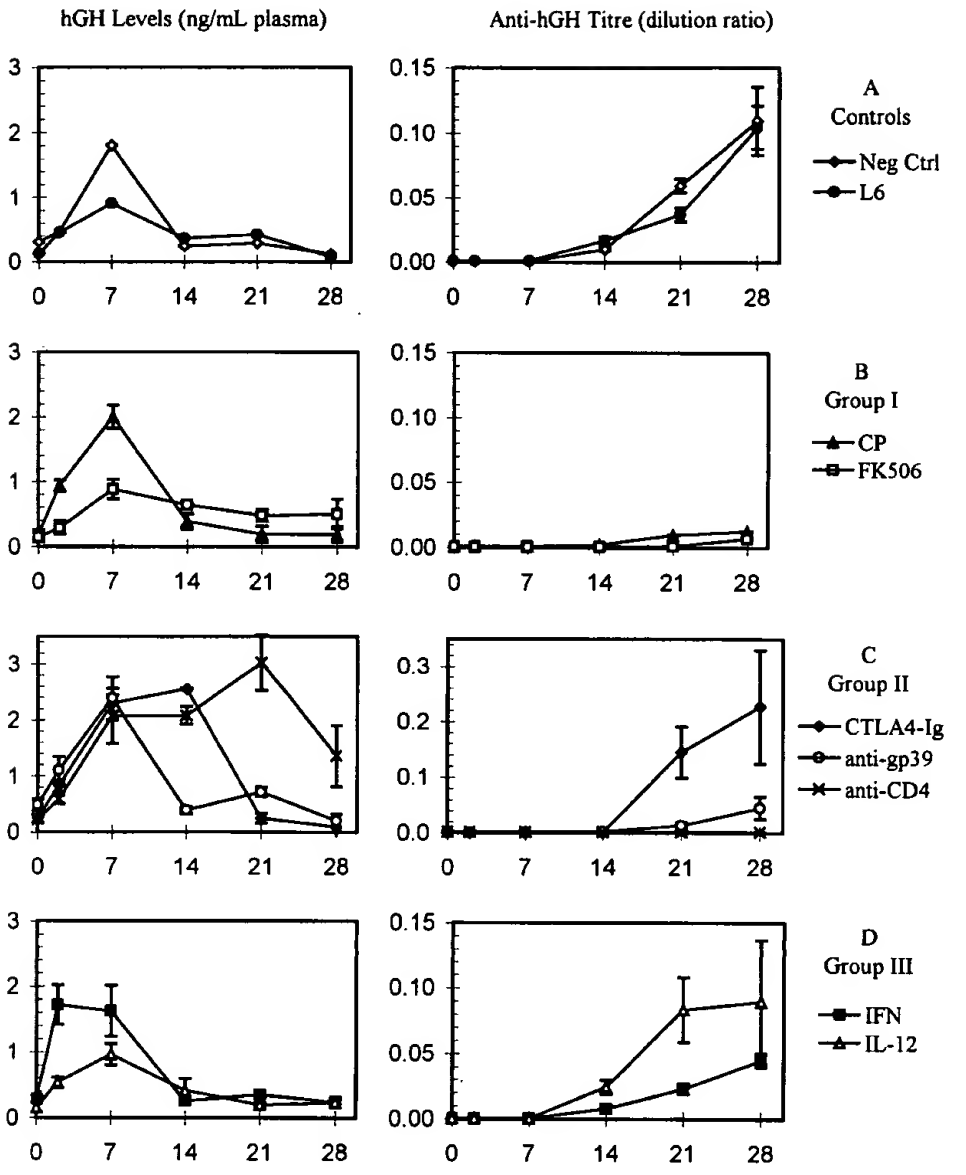

FIGURE 2. *In vivo* comparison of plasma hGH and anti-hGH antibody levels. Each animal was implanted with encapsulated hGH-secreting myoblasts on day 0 and treated with one of the immunosuppressive agents. The control groups included the negative control group, which were implanted with encapsulated hGH-secreting cells but received no immunosuppression, and the L6 group, which was similarly implanted but was treated with an irrelevant fusion protein. Group I included the immunosuppressive drug treatments (CP: cyclophosphamide; FK506), Group II the anti-T-cell receptor antibody treatments (CTLA4-Ig; anti-gp39; antiCD4) and Group III the cytokine treatments (IFN: interferon-γ; IL-12: interleukin 12). On various days post-implantation, the levels of hGH (ng/mL plasma) and titers of anti-hGH antibodies were determined. The anti-hGH antibody titer was ex-

Group II

These mice were treated with antibodies directed against various T-cell receptors. Their responses were highly variable depending on the antibody used (FIG. 2C, left panel). The CTLA4-Ig treated mice had a peak level of hGH on day 14 with 2.5 ng/mL before declining to near zero on day 28. CTLA4-Ig is a fusion protein that acts as a competitive inhibitor of CD28. It prevents stimulation of T cells by blocking the T-cell CD28 receptor from interacting with ligands on antigen presenting cells.[17, 18] In experimental systems, its administration results in suppression of the inflammatory response with the inhibition of T_{H1} cytokines (IL-2 and IFN-γ) but not T_{H2} cytokines (IL-4, IL-10 and IL-13).[19] The elevation in anti-hGH antibody titer to twice that of its control L6, an irrelevant fusion protein (FIG. 2C), indicates that the T_{H2} response to hGH was probably more important than the T_{H1} response to mediate B-cell expansion and antibody production. Similarly, the anti-gp39–treated mice showed a peak delivery of hGH at 2.4 ng/mL on day 7, which declined to background level by day 28, a pattern that was almost identical to the controls. In contrast, the anti-CD4–treated mice had a much more sustained delivery, rising to the same level as the controls on day 7 (~2 ng/mL) but continuing to increase to 3 ng/mL by day 21 before declining by day 28 to 1.4 ng/mL (a level that was still statistically greater than the controls, $p < 0.05$).

The immune response to hGH was also highly variable in this group (FIG. 2C, right panel). The CTLA4-Ig–treated mice had a delayed but exaggerated antibody response, the titer rising sharply only on day 21 and continuing to escalate until day 28 to a level about 200% of its controls, which were implanted mice treated with an irrelevant antibody L6 (FIG. 2A, right panel). In contrast, the anti-gp39–treated animals showed a moderate suppression compared to the controls with a slight rise in titer by day 14 which then escalated during the remaining 14 days to about 35% of the control level. The anti-gp39 antibodies are thought to block the interaction between the gp39 protein and the CD40 receptor expressed on all mature B-cells. Blockage of CD40 and gp39 interactions via antibodies to gp39 is thought to prevent initiation of B-cell proliferation and antibody class switching. It has been shown to reduce both primary and secondary humoral immune response in mice when administered with soluble protein antigens, achieving >90% antibody suppression.[20] However, in our current protocol, anti-gp39 treatment only modestly lowered the anti-hGH antibody titer (42 ± 19% of control, FIG. 3), and was insufficient to influence hGH delivery *in vivo* (FIG. 2C). The anti-CD4 group showed the most effective suppression of anti-hGH response. Throughout the 28 days of the experiment, there was very little detectable titer of anti-hGH antibodies beyond background (FIG. 2C, right panel). The anti-CD4 antibody has been used to transiently deplete CD4 cells (T-helper cells) to prevent neutralizing antibody formation in recombinant gene therapy.[21] This treatment almost completely suppressed antibody response to hGH (1.2 ± 0.6% of the control, FIG. 3), and also allowed sustained circulation of hGH even up

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pressed as the dilution of a positive control plasma (mice specifically immunized with purified hGH) to contain an equivalent amount of anti-hGH antibodies. All data are expressed as the mean ± standard error (N = 4 per treatment except for FK506 and IFN-γ where N = 3). (From Potter *et al.* 1998,[26] reprinted with permission.)

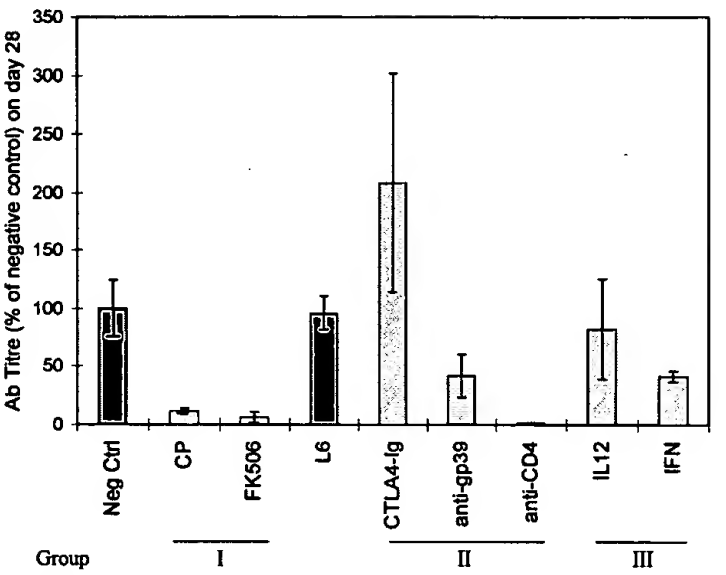

FIGURE 3. Comparison of antibody titers on day 28 post-implantation. Day 28 antibody titers against hGH were measured in plasma from animals treated with the seven immunosuppressive protocols (as described in FIG. 2) and the group mean expressed as a percent of the negative control group ± standard error. (From Potter *et al.* 1998,[26] reprinted with permission.)

to day 28 at levels of 1 ng/mL of plasma. This result agreed with those obtained by Yang,[21, 22] who used anti-CD4 antibodies to suppress adenovirus vector–neutralizing antibodies and to improve transgene delivery. Therapeutic approaches using anti-CD4 antibodies have already been designed in humans for the treatment of transplant rejection and autoimmune diseases,[23] suggesting that anti-CD4 treatment may be a promising agent for use in human gene therapy protocols.

Group III

These mice were treated with the cytokines IL-12 or IFN-γ. Their circulating levels of hGH were similar to that of the control group at 1.0 and 1.6 ng/mL on day 7, respectively (FIG. 2D, left panel). Although they both showed an antibody response by day 14, IFN-γ appeared partially successful in suppressing the anti-hGH response, with titers rising to less than half of the control levels (41%) by day 28, whereas the IL-12 treated mice had a similar level of response as the controls with an even earlier peak at day 21 (FIG. 2D, right panel). IL-12 is a cytokine that activates T_{H1} cells at the expense of T_{H2} activation, which are thought to be more important in broader antibody responses, inhibiting the production of antibodies.[24] IL-12 is expected to suppress the anti-hGH IgG response better than CTLA4-Ig, since CTLA4-Ig only blocks the T_{H2} response by inhibiting the second signal required for T_{H2} cell stimulation. Although this was indeed observed (FIGS. 2C and 2D), the suppression by IL-12 was not low enough to reduce it from those of the controls (FIG. 2A). IL-12 has been successfully used to reduce neutralizing (IgA) antibodies and improve transgene delivery, but these therapies had no effect on IgG levels in lung tissue.[22]

In another experiment targeting liver cells, IL-12 achieved only a partial reduction in neutralizing antibody levels insufficient to improve transgene expression.[21] Hence, it is likely that both T_{H1} and T_{H2} response were important in the mounting of antibody response against hGH delivered continuously by implanted cells. Suppression of either one alone was thus ineffective. Interferon-γ is another cytokine secreted by activated macrophages and T-helper cells. It is thought to mediate the effect of IL-12 and thus achieves the same immune suppression[24] of antibody production by activating T_{H1} cells to promote cytotoxic lymphocyte and IgG_{2A} antibody response. However, although IL-12 treatment had little effect on antibody titer (82 ± 43% of control), IFN-γ actually achieved a modest suppression of the antibody response, at 41 ± 5% of the control (FIG. 3), thus indicating the complexity of the immune response.

Hence, the efficacy of the different treatment protocols in suppressing the antibody response to a foreign protein appeared to be highly variable. While the differences between most of the groups were not statistically significant by ANOVA, there was a trend of decreasing suppression in the order: anti-CD4 > FK506 > cyclophosphamide > anti-gp39 = IFN-γ, while IL-12 and CTLA4-Ig were not effective (FIG. 3). In particular, the suppression by anti-CD4 was almost 100% with little detectable antibody to hGH while that of FK506 and cyclophosphamide was ≥ 90% compared to the controls.

LOSS OF GENE PRODUCT DELIVERY

With the exception of the anti-CD4–treated animals, all other groups showed a loss of hGH by day 28 post-implantation. In general, this loss was accompanied by a corresponding rise in antibody titer. It was postulated that the lack of detectable circulating hGH was either due to clearance of hGH from the plasma by anti-hGH antibodies[12] or the loss of microcapsule function after implantation. To investigate the first possibility, we injected 6 mg of purified hGH into each of three mice from a group previously implanted with microcapsules and hence sensitized to hGH (CTLA4-Ig–treated mice). No hGH could be detected in the plasma of any of these implanted mice, even at 1 minute after the hGH injection (FIG. 4). However, when four naïve mice which had not received any prior microcapsule implantations were similarly treated, they showed a high level of hGH immediately after the injection, which continued to be measurable at a level of 75 ng hGH/mL plasma at 30 minutes after the injection (FIG. 4). Since the level of hGH expression at implantation was 83 ± 24 ng/10^6 cells/h and 1.26 ± 0.36 × 10^6 cells were implanted, the maximal amount of hGH delivered *in vivo* was expected to be 104 ng/h. Because the half-life of hGH in mice is only 2.2 ± 0.5 min,[25] such a level of delivery would have been readily cleared from the circulation by the sensitized mice, thus accounting for the loss of detectable hGH.

To verify that the loss of detectable hGH was not due to breakdown of microcapsules or loss of transgene expression, representative animals from all groups were sacrificed on day 28 to recover the microcapsules. The microcapsules appeared intact and free within the abdominal cavity. When compared to the encapsulated cells kept *in vitro*, the viability of those retrieved after IP implantation was quite similar,

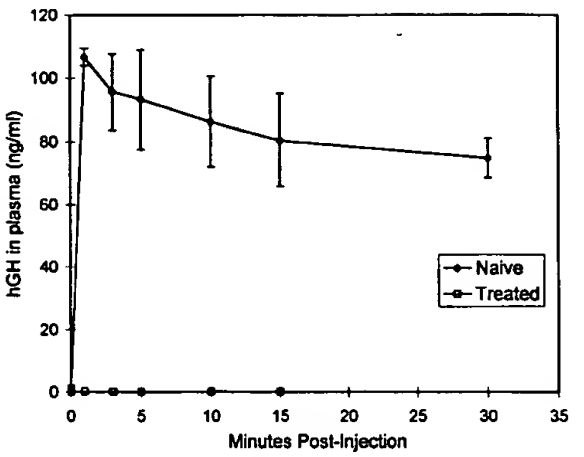

FIGURE 4. Clearance of hGH by naïve vs. treated mice. Naïve mice never exposed to hGH (N = 4) and treated mice that had been implanted with microcapsules secreting hGH two months prior to the clearance study (N = 3) were each injected with 6 μg hGH IV at time 0. Blood was sampled at the times shown post-injection and assayed for hGH in the plasma. Data are expressed as the mean ± standard error. (From Potter *et al.* 1998,[26] reprinted with permission.)

ranging from a low of 69% for the L6 control mice to a high of 94% for the FK506 treated mice, with an average viability for all retrieved capsules of 83% (TABLE 2). There was no statistically significant difference in cell viability among the different treatment groups. However, compared to the microcapsules kept *in vitro*, those retrieved after 28 days of implantation showed a vastly increased cell density. As shown in TABLE 2, the retrieved capsules had cell numbers ranging from approximately 1000/capsule for the negative control mice to about 2500/capsule for the IL-12 treated mice, with an average of around 1600/capsule for all the retrieved capsules. This represented an increase of fivefold in cell density compared to the capsules kept *in vitro* (approximately 300 cells/capsule) for the same duration.

Secretion rates of hGH from the capsules after retrieval on day 28 were similar to those of capsules kept *in vitro* until day 28 (FIG. 5), i.e., no significant difference between groups by ANOVA. The average hGH secretion from recovered capsules for the nine treatment and control groups was 44 ng/10^6 cells/h, which represented an overall decrease in secretion from the pre-implantation level of 83 ± 24 ng/10^6 cells/h, possibly owing to the removal of G418-selection pressure *in vivo*.

CONCLUSION

The responses of the recipients to the various treatments can be classified into three categories. The first category, shown by treatment with CTLA4-Ig or interleukin-12, was similar to the untreated controls: no suppression of anti-hGH antibodies and no significant improvement in delivery of hGH. The second category of response observed in four treatment protocols (cyclophosphamide, FK506, anti-gp39 and interferon-γ), was suppression of antibodies but no improvement in sustaining delivery

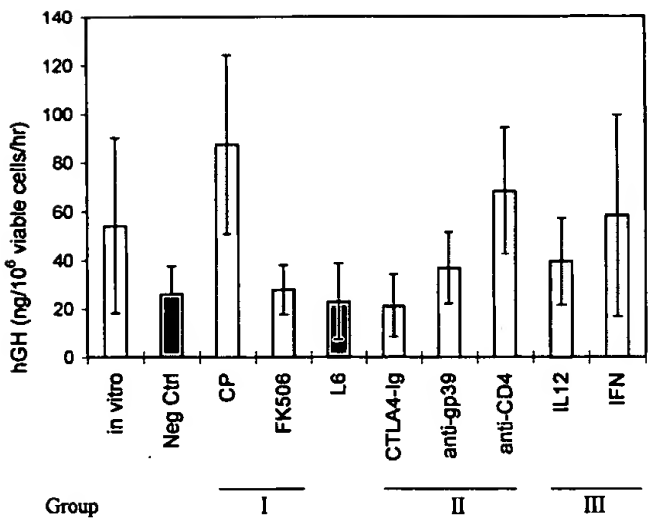

FIGURE 5. hGH secretion from retrieved capsules after day 28 of implantation. On day 28 post-implantation, microcapsules were retrieved from the intraperitoneal cavity by lavage, washed, and kept under the usual tissue culture conditions to determine the rate of hGH secretion (see METHODS). The microcapsules were then ruptured to determine the viable cell number. The data represented mean secretion ± S.D. The *in vitro* group refers to non-implanted microcapsules kept *in vitro* for 28 days under the usual tissue culture conditions (see FIG. 2 for remainder of legend). (From Potter *et al.* 1998,[26] reprinted with permission.)

of transgene product (FIG. 2). It is clear that while these treatments are more effective in antibody suppression than CTLA4-Ig and IL-12, they were unable to exert sufficient suppression of the humoral response to permit a sustained level of circulating recombinant hGH produced by the encapsulated cells. The last category of response was seen in the group of mice receiving anti-CD4: strong antibody suppression and the most sustained hormone delivery.

In conclusion, of the seven immune suppressive reagents, the use of anti-CD4 that interferes with T-helper cell response and B-cell activation appears most promising. It allowed prolonged delivery of a foreign protein and suppression of antibody response to a recombinant gene product. Hence, its role as adjunct treatment for appropriate patients at risk for developing inhibitors merits further consideration.

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